भारतीय मानक
Indian Standard

IS 7874 (Part 3) : 2020

पशु आहार एवं पशु आहार सामग्री की परीक्षण पद्धतियाँ

भाग 3 सूक्ष्मजीवविज्ञानी पद्धतियाँ

(पहला पुनरीक्षण)

Methods of Tests for Animal Feeds and Feeding Stuffs

Part 3 Microbiological Methods

(First Revision)

ICS 65.120

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FOREWORD

This Indian Standard (Part 3) (First Revision) was adopted by the Bureau of Indian Standards, after the draft finalized by the Animal Husbandry, Feeds and Equipment Sectional Committee had been approved by the Food and Agriculture Division Council.

The importance of adoption of standard and uniform methods of analysis for quality control purposes needs no emphasis. Such methods not only help reducing divergence in the analytical results but also ensure and enable a proper comparison and correct interpretation of the test results.

The use of compounded feeding stuff and supplements is growing fast. Therefore availability of such material duly tested for their quality and safety assumes higher importance. A series of Indian Standards have been published, which include methods of test of the full range of animal feeds. This standard (Part 3) covers the methods of detecting and identifying pathogenic organisms, the presence of which in the feed make it unfit for consumption by animals.

This standard (Part 3) was first published in 1975 and is being revised in order to forestall the use of laboratory animals in confirmatory tests. The new methods incorporated in this standard are based on Polymerase Chain Reaction (PCR), which are not only very precise and sensitive but also preclude the use of guinea pigs for laboratory testing.

This Indian Standard is published in three parts. The other parts in this series are:

Part 1 General methods

Part 2 Minerals and trace elements

Significant assistance has been obtained from the documents published by World Health Organization (WHO) on this subject.

In reporting the result of a test or analysis made in accordance with this standard, if the final value, observed or calculated, is to be rounded off, it shall be done in accordance with IS 2 : 1960 ‘Rules for rounding off numerical values (*revised*)’.

Indian Standard

METHODS OF TESTS FOR ANIMAL FEEDS AND FEEDING STUFFS

PART 3 MICROBIOLOGICAL METHODS

(First Revision)

1 SCOPE

This standard (Part 3) prescribes the microbiological methods for testing animal feeds and feeding stuffs.

2 REFRENCES

The standards given below contain provisions which, through reference in this text, constitute provisions of this standard. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this standard are encouraged to investigate the possibility of applying the most recent editions of these standards.

IS No.	*Title*
1070 : 1992	Regent grade water (*third revision*)
5887	Methods for detection of bacteria responsible for food poisoning
(Part 3) : 1999	General guidance on methods for detection of *Salmonella* (*second revision*)
(Part 4) : 1999	Isolation and identification of *Clostridium perfringens* (*Clostridium welchii*) and *Clostridium botulinum* and enumeration of *Clostridium perfringens* (*second revision*)

3 QUALITY OF REAGENTS

Unless specified otherwise, pure chemicals and distilled water (*see* IS 1070) shall be employed in tests.

NOTE — 'Pure chemicals' shall mean chemicals that do not contain impurities which affect the test result.

4 SAMPLING

During collection of samples, strict precautions should be taken to avoid contamination. If feeds are packaged, an effort should be made to obtain the sealed packages. For bulk feed, samples may be collected in sterile plastic bags or sterile screw-capped jars. A sample of at least 100 g should be obtained for tests.

5 TESTS FOR *BACILLUS ANTHRACIS*

5.1 General

The method involves detection of *Bacillus anthracis* from animal feeds and/or feed supplements by isolation of test organism and identification by microbiological and biochemical tests. Confirmation of test organism shall be done by the methods given in **5.5.3**.

NOTE — Direct confirmation through PCR based test method as given in **5.5.3.2** precludes the necessity of carrying out morphological and biochemical tests.

5.2 Culture Media and Reagents

5.2.1 *Polychrome Methylene Blue*

Prepare as follows:

Dissolve 0.2 g methylene blue in 10 ml absolute alcohol. Add 90 ml distilled water to it. Filter the solution through filter paper. The stain is kept in half filled bottles for proper aeration and thereby ripening. Store at room temperature in cotton plugged half-filled bottle at least for few months with intermittent shaking for maturation of stain. For good quality maturation, storage of 10 years is recommended. A matured stain is called polychrome methylene blue. Add 1 percent potassium carbonate to the stain to hasten the ripening process.

5.2.2 *Polymyxin-Lysozyme-EDTA-Thallous Acetate (PLET) Agar*

5.2.2.1 *Composition*

EDTA	:	0.3 g
Thallous acetate	:	0.4 g
Agar	:	15 g
Distilled water	:	1 000 ml

5.2.2.2 *Preparation*

Mix the constituents and sterilize by autoclaving at 121°C for 15 min. Cool to 50°C and add the following:

a) Polymyxin B sulphate : 30 000 Units
b) Lysozyme : 3 00 000 Units

5.2.3 *Bi-Carbonate/Serum Agar*

Prepare as follows:

Add 0.3 g yeast extract, 0.5 g glucose and 2.5 g agar in 100 ml of nutrient broth. Autoclave at 115°C for 10 min, cool to 55°C and add filter sterilized 11 ml each of 7 percent $NaHCO_3$ and 7 percent BSA fraction V to have 0.7 percent final concentration of each. Mix and pour the plate. Instead of BSA fraction V, any filter sterilized serum can be added at a rate of 20 percent (*v/v*) final concentration.

5.2.4 *Tryptic Soy Broth*

5.2.4.1 *Composition*

Casein digest peptone	:	17 g
Papaic digest of soybean meal	:	3 g
Disodium phosphate	:	2.5 g
Dextrose	:	2.5 g
Sodium chloride	:	5 g
Distilled water	:	1 000 ml

Adjust the *p*H to 7.3 ± 0.2 and then sterilize it at 121°C for 15 min.

5.2.5 *Nutrient Gelatin Medium*

5.2.5.1 *Composition*

Peptone	:	5.0 g
Beef extract	:	3.0 g
Gelatin	:	120.0 g
Distilled water	:	1 000 ml

Adjust the *p*H to 6.8 ± 0.2 and then sterilize it at 121°C for 15 min.

5.2.6 *Phenol Red Salicin Broth*

5.2.6.1 *Composition*

Trypticase	:	10 g
Sodium chloride (NaCl)	:	5 g
Beef extract (optional)	:	1 g
Phenol red	:	0.018 g
(7.2 ml of 0.25 percent phenol red solution)		
Salicin	:	10 g
Distilled water	:	1 000 ml

5.2.6.2 *Preparation*

Dissolve the ingredients. Autoclave it at 121°C for 3 min only. Broth medium will be of light red colour. Adjust the *p*H to 7.4 ± 0.2 and then autoclave it at 121°C for 15 min.

5.2.7 *Litmus Milk Broth*

5.2.7.1 *Composition*

Skimmed milk powder	:	100 g
Litmus	:	0.075 g
Distilled water	:	1 000 ml

Adjust the *p*H to 6.8 ± 0.2 and then sterilize it at 121°C for 15 min.

5.2.8 *Egg Yolk Agar*

5.2.8.1 *Composition*

Proteose peptone	:	40 g
Disodium phosphate	:	5 g
Monopotassium phosphate	:	1 g
Sodium chloride	:	2 g
Magnesium sulphate	:	0.1 g
Glucose	:	2 g
Hemin	:	0.005 g
Agar	:	25 g
Distilled water	:	1 000 ml

Adjust the *p*H to 7.6 ± 0.2 and then sterilize it.

5.2.8.2 *Preparation*

Dissolve the ingredients. Sterilize by autoclaving 121°C for 15 min. Cool to 45-50°C and add 10 ml of sterile egg yolk emulsion per 90 ml of medium.

5.2.9 *Ethidium Bromide Stock Solution (10 mg/ml)*

Prepare as follows:

Ethidium bromide	:	100 mg
Distilled water	:	10 ml

Mix the solution and store at 4ºC. A concentration of 1.0 µg/ml should be used in preparing agarose gel (for example, if agarose gel of 50 ml volume is prepared, then add 5 µl of stock solution of ethidium bromide that is 10 mg/ml).

5.2.10 *Tris-Acetate-EDTA (TAE) Buffer (50 X Stock Solution)*

Prepare as follows:

5.2.10.1 *Composition*

Tris base	:	24.2 g
Glacial acetic acid	:	5.71 ml
EDTA (0.5 M; *p*H 8.0)	:	10 ml
Add distilled water to make	:	100 ml

Prepare a working solution of 1 X solution by appropriate dilution.

5.2.11 *Sheep Blood Agar*

Prepare as follows:

Add sterile de-fibrinated blood (5 to 10 percent by mass), collected aseptically from a healthy sheep, to nutrient agar that has been melted and cooled to 48°C. Mix well and pour into sterile plates with aseptic precautions before flame and allow to set. Incubate overnight at 37°C to check for sterility.

5.3 Other Reagents

5.3.1 *Sterile Sodium Chloride Solution (Normal Saline)* — 0.9 percent (*m/v*)

5.3.2 *Gram's Staining Kit*

5.3.3 *PCR Grade Water*

5.3.4 *PCR Chemicals*

5.3.5 *Agarose (1 Percent)*

5.4 Apparatus

5.4.1 *Biosafety Level 3 (BSL-3) Cabinet — Bacillus anthracis* is a BSL-3 pathogen and hence the entire feed sample processing work, such as isolation, smears preparation for staining, biochemical tests and PCR DNA template preparation shall be carried out in BSL-3 cabinet.

5.4.2 *Weighing Balance*

5.4.3 *Water Bath*

5.4.4 *Conical Flasks, Beakers*

5.4.5 *Petri Plates Bacteriological Streaking Loop*

5.4.6 *Bacteriological Incubator*

5.4.7 *Microscope*

5.4.8 *Microscopic Slides*

5.4.9 *Cover Slips*

5.4.10 *Ice Bath*

5.4.11 *PCR Tubes of 0.2 ml*

5.4.12 *Microfiltertips of Capacities 10, 100 and 1 000 µl*

5.4.13 *Variable Micropipettes of Capacities 0.2. 10, 100 and 1 000 µl*

5.4.14 *PCR Machine — For running PCR master mix reaction*

5.4.15 *Horizontal Electrophoresis System Gel Documentation System*

5.4.16 *Hot Air Oven*

5.4.17 *Autoclave*

5.5 Procedure

5.5.1 *Isolation of Test Organism and Microscopic Examination*

Any of the following methods may be followed.

5.5.1.1 *Method A*

Shake about 20 g of the material with 200 ml of sterile sodium chloride solution. Decant the supernatant. Centrifuge and suspend the packed sediment in about 3 ml of the sterile sodium chloride solution. Heat for 10 min at 70°C in a water bath. Streak on at least three blood agar plates from this suspension and incubate at 37°C for 24 h. Examine the characteristic colonies of *Bacillus anthracis*. The colonies are circular and disc-like, white granular, about 3 mm in diameter, showing a ground- glass appearance and a wavy margin with only a very slight zone of haemolysis.

5.5.1.2 *Method B*

Shake about 20 g of the material with 200 ml of sterile sodium chloride solution. Decant the supernatant. Centrifuge and suspend the packed sediment in about 3 ml of the sterile sodium chloride solution. Heat it for 10 to 15 min at 65 to 70°C in a water bath. Prepare tenfold dilution of the samples ranging from 1 : 10 to 1 : 100 and streak all the dilutions in duplicate onto sheep blood agar and PLET agar plates. Incubate the sheep blood agar plates at 37°C for 24 h and PLET agar plates for 36-48 h. Examine microscopically. The colonies of *Bacillus anthracis* on sheep blood agar are flat or slightly convex, irregularly round, with edges that are slightly undulated (irregular, wavy border), and have a ground-glass appearance. There may be often comma-shaped projections from the colony edge, producing the medusa-head colony. Colonies on PLET agar will be smaller than that on blood agar, with roughly circular, creamy white and ground-glass texture. Examine further, microscopically after gram staining. Prepare thin smears out of the cultures on clean grease-free microscopic glass slides. Air dry the smear. Fix it by dipping in absolute alcohol for 30-60 s and re-dry or heat fix. Flood the fixed smear using crystal violet. Keep it for 1 min. Rinse off the crystal violet using tap water. Flood the slide with iodine solution. Keep for 1 min. Rinse off the iodine solution using tap water. Decolorize the slide for 10 s using 95 percent alcohol till the pink colour ceases off. Wash off the slide and completely drain. Counter-stain the slide using saffranin or basic fuchsin for 30 s. Drain and blot dry. Observe the slide under oil immersion objective of the microscope. Presence of Gram-positive, rod-shaped bacteria, 3 to 8 µm × 1 to 1.2 µm in

size, straight or slightly curved with square or truncated ends with parallel sides found usually single, in pairs or chains of 3 or 4 bacilli, arranged characteristically in bamboo stick appearance is indicative of *Bacillus anthracis*.

5.5.2 *Biochemical Tests*

The tests described in **5.5.2.1** to **5.5.2.7** are used to assist in the differentiation of *Bacillus anthracis* from the anthrax-like or Pseudo-anthrax bacilli. The biochemical reactions in different tests are given in Table 1. The culture of the suspect colonies obtained by using the PLET Agar in method B at **5.5.1.2** shall be used.

5.5.2.1 *Motility test*

Any of the following two methods may be followed.

5.5.2.1.1 Transfer 2 drops (approximately 0.1 ml) of tryptic soy broth, or equivalent, into a sterile glass tube. Using an inoculating loop, transfer a portion of the suspect colony from a 12-20 h culture and suspend the growth in the broth medium. Alternatively, a loopful of medium from a fresh broth culture can be used. Transfer 10 µl of the suspension to a microscope slide and overlay with a coverslip. Examine slide under a microscope using the 40X objective (total magnification 400X) (may also be viewed at 1 000 X with oil objective). Observe for motility, which is indicative of *Bacillus anthrax*. The test shall be performed only in BSL-3 cabinet.

5.5.2.1.2 Using a sterile inoculating needle, remove a portion of growth from an isolated, suspect colony after 18-24 h incubation. Inoculate the motility medium by carefully stabbing the needle 3-4 cm into the medium and then drawing the needle directly back out so that a single line of inoculum can be observed. Incubate the tube at 35-37°C in ambient atmosphere for 18-24 h. Observe for motility which is reflected in the form spreading of culture around the side of inoculation, which is indicative that the test is negative for *Bacillus anthrax*, since they are non-motile. Prepare the motility medium as given below:

Tryptose	:	10.0 g/l
Sodium chloride	:	5.0 g/l
Agar	:	5.0 g/l
Final *p*H (at 25°C)	:	7.2 ± 0.2

Directions — Suspend all ingredients in 1 000 ml distilled water. Heat to boiling to dissolve the medium completely. Dispense in tubes (5 to 10 ml/tube) and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 min. Allow tubed medium to cool to 45-50°C in an upright position.

5.5.2.2 *Haemolysis on sheep blood agar*

Examine the suspect colonies developed on sheep blood agar (*see* **5.2.11**) for hemolysis, the absence of which is indicative of *Bacillus anthracis*.

5.5.2.3 *Methylene blue reduction*

Examine the suspect colonies (*see* **5.5.1.2**) for reduction of methylene blue after 24 h. Non- reduction or slight reduction of methylene blue is indicative of *Bacillus anthrax*.

5.5.2.4 *Fermentation of salicin*

Aseptically inoculate each test tube containing phenol red salicin media (*see* **5.2.6**) with the test microorganism using an inoculating needle or loop. Swirl the tube gently to mix contents. Avoid contact of liquid with tube cap. Incubate tubes at 35 to 37°C for 18 to 24 h. Lack of fermentation is indicative of *Bacillus anthracis*. Longer incubation periods may be required to confirm a negative result.

5.5.2.5 *Gelatin liquefaction*

A heavy inoculum of an 18-24 h old test bacteria is stab-inoculated into tubes containing nutrient gelatin

Table 1 Tests to Differentiate *Bacillus anthracis* from the Anthrax Like or Pseudo-anthrax Bacilli

(*Clause* 5.5.2)

Sl No.	Tests	B. anthracis	Anthrax Like or Pseudo Anthrax Bacilli
(1)	(2)	(3)	(4)
i)	Motility	Non-motile	Majority motile
ii)	Haemolysis on sheep blood agar	None or very slight	Mostly beta haemolytic type
iii)	Methylene blue reduction	Not reduced or slightly reduced in 24 h	Usually reduced in 24 h
iv)	Salicin fermentation	Usually negative or late	Often positive in 24 h
v)	Gelatin liquefaction (7 days)	Negative or partial	Usually complete
vi)	Litmus milk	Not reduced or slowly	Usually reduced in 2-3 days; No peptonisation
vii)	Lecithinase activity	If produced, weakly positive	Not produced (except *B. cereus*)

medium (*see* **5.2.5**). The inoculated tubes along with an un-inoculated control tube is incubated at 37°C for up to 1 week, checking every day for gelatin liquefaction. Gelatin normally liquefies at 28°C and above so to confirm that liquefaction was due to gelatinase activity, the tubes are immersed in an ice bath for 15-30 min. The tubes are tilted to observe if gelatin has been hydrolyzed. Hydrolyzed gelatin will result in a liquid medium even after exposure to cold temperature (ice bath) while the un-inoculated control medium will remain solid. The hydrolysis of gelatin indicates the secretion of gelatinase by the test organism into the medium which indicates that the test is negative for *Bacillus anthracis*.

5.5.2.6 *Litmus milk fermentation test*

Obtain two litmus milk broths (*see* **5.2.7**) and inoculate one broth with test culture. Leave the other broth un-inoculated (this will act as a negative control). Incubate both broths at 37°C. Observe the broth cultures over the next several days. Changes should be visible in five to seven days. Non reduction of litmus milk is indicative of *Bacillus anthracis*.

5.5.2.7 *Lecithinase activity*

Inoculate the samples in egg yolk agar (*see* **5.2.8**). Incubate at 35-37°C for 18-48 h. Examine for the slight opalescent growth on agar which is indicative of *Bacillus anthracis* and if the growth is intense, it is positive for *Bacillus cereus*.

5.5.3 *Confirmatory Tests*

5.5.3.1 *Polychrome methylene blue staining*

Induce capsule formation of bacilli by the following methods.

5.5.3.1.1 *In blood*

Transfer a pinhead quantity of growth from a suspect colony to 2.5 ml defibrinated sheep or horse blood in a sterile test tube or small bottle. Incubate for 5-18 h at 37°C. Make a thin smear on a microscope slide by transferring the incubated material from the bottom of unshaken bottle or tube, using a 1 ml loop.

5.5.3.1.2 *On bicarbonate/ serum agar plates*

Plate the suspect colony onto bicarbonate/serum agar (*see* **5.2.3**). Incubate overnight at 37°C under a 10-20 percent CO_2 atmosphere. Make a thin smear on microscope slide.

NOTE — Although the capsule stains well when produced by this method, it does not appear so well circumscribed as when produced in vivo or in blood as described above.

Place a large drop of polychrome methylene blue over the smear to cover it completely. Leave to air dry for 30-60 s. Wash off the slide with 10 000 ppm hypochlorite solution. Examine under the oil immersion objective of the microscope. Presence of capsulated bacilli (pink capsule surrounding dark blue bacilli, often square shaped, singly or in short chains) is definitive for *Bacillus anthracis*.

5.5.3.2 *Polymerase chain reaction method*

5.5.3.2.1 *Preparation of DNA template*

Pick two to three colonies and suspend in sterile normal saline. Adjust OD of culture to 1.0 McFarland units. 200 µl of this suspension is taken for DNA extraction, which is carried out as per the instruction provided by manufacturer's kit.

5.5.3.2.2 *Primers used*

The primers for confirming *Bacillus anthracis* are given in Table 2.

5.5.3.2.3 *PCR master mix*

Carry out PCR using 25 µl reaction volume. PCR mix is as follows:

PCR buffer	:	2.5 µl
$MgCl_2$	:	2.5 µl
DNTPs	:	4.0 µl
Primer F	:	1 µl (50 pico mole)
Primer R	:	1 µl (50 pico mole)
Taq polymerase	:	0.25 µl
DNA Template	:	5 µl
PCR Grade water	:	to make the 25 µl reaction volume

Table 2 Primers for Confirming *Bacillus anthracis*

(*Clause* 5.5.3.2.2)

Target Gene	Primer Sequence 5' – 3'	Product Size (bp)	Reference
PA	F-TCC TAA CAC TAA CGA AGT GAA GGA	569	WHO (2003)
	R- CTG GTA GAA GGATAT ACG GT		
CAP	F- CTG AGC TAA TCG ATA TG	846	WHO (2003)
	R- TCC CAC TTA CGTAAT CTG AG		

5.5.3.2.4 *PCR cycling conditions*

Use the following PCR cycles: 1 × 95°C for 5 min; 30 × (95°C for 0.5 min, followed by 55°C for 0.5 min, followed by 72°C for 0.5 min); 1 × 72°C 5 min; cool to 4°C:

Initial Denaturation	Denature	Anneal	Extend	Final extension	Hold
	30 cycles				
95°C for 5 min	95°C for 30 s	55°C for 30 s	72°C for 30 s	72°C for 5 min	4°C

After PCR, 10 percent of the reaction volume of tracking dye (0.02 percent xylenecyanol, 0.02 percent bromophenol blue and 50 percent glycerol) is added to each PCR tube, and 10 µl loaded into wells on a 1 percent agarose gel in TAE and the gel electrophoresed for 45 min at 120 V followed by staining in ethidium bromide solution for visualization under UV light. A 1-kilobase ladder for size markers should be included in the outer walls of the gel. A known virulent *B. anthracis* clinical strain for PA and CAP or alternatively *B. anthracis* Sterne or Pasteur strain for PA gene only. Negative control: Non-pathogenic *Bacillus* spp., *B. subtilis* / *B. cereus*.

The PCR products showing bands of amplicon size corresponding to the primers used indicate sample positive for *Bacillus anthracis* (*see* Fig. 1).

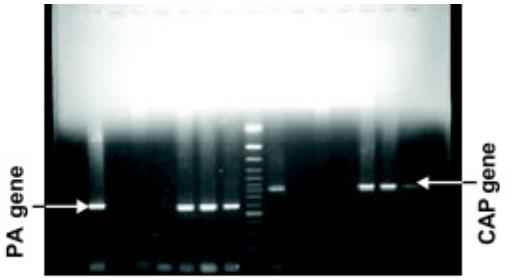

FIG. 1 GEL PICTURE OF PCR PRODUCTS FOR THE DETECTION OF *B. ANTHRACIS*

6 TESTING FOR *CLOSTRIDIUM SP.*

6.1 General

The method involves detection of *Clostridium sp.* from animal feeds and/or feed supplements by isolation of the organism, identification through microscopic examination, and confirmation of test organism by polymerase chain reaction.

6.2 Media, Reagents and Chemicals

6.2.1 *Sheep Blood Agar*

Prepare as follows:

Add sterile defibrinated blood (5 to 10 percent by mass), collected aseptically from a healthy sheep to nutrient agar that has been melted and cooled to 48 °C. Mix well and pour into sterile plates with aseptic precautions before flame and allow to set. Incubate overnight at 37°C to check for sterility.

6.2.2 *Trypticase Peptone Glucose Yeast Extract (TPGY) Medium*

Prepare as follows:

Trypticase	:	50 g
Peptone	:	5 g
Yeast extract	:	20 g
Dextrose	:	4 g
Sodium thioglycolate	:	1 g
Distilled water	:	1 000 ml

Dissolve solid ingredients of base and autoclave for 15 min at 121°C. Adjust final *p*H to 7.0 ± 0.2 and refrigerate at 5°C. Add 67 ml of trypsin solution [1.5g trypsin (1:250) powder in 100 ml distilled water, filter and sterilize] to get a final concentration of 0.1 percent, immediately before use.

6.2.3 *Sterile Sodium Chloride Solution (Normal Saline)* **—** 0.9 percent (*m/v*).

6.2.4 *PCR Grade Water* **—** For PCR master mix preparation.

6.2.5 *PCR Chemicals* — PCR master mix preparation.

6.2.6 *Agarose (1 Percent)* — For resolving amplified PCR products.

6.2.7 *Tris-Acetate-EDTA (TAE) Buffer (50X Stock Solution)*

Prepare as follows:

Tris base	:	24.2 g
Glacial acetic acid	:	5.71 ml
EDTA (0.5 M; *p*H 8.0)	:	10 ml
Add distilled water to make	:	100 ml

A working solution of 1 X solution should be made from 50X stock solution by appropriate dilution with distilled water.

6.2.8 *Ethidium Bromide Stock Solution (10 mg/ml)*

Prepare as follows:

Ethidium bromide	:	100 mg
Distilled water	:	10 ml

The solution should be mixed and stored at 4ºC. A concentration of 1.0 µg/ml should be used for preparing agarose gel.

6.3 Apparatus

6.3.1 *BSL-3 Cabinet*

6.3.2 *Weighing Balance*

6.3.3 *Water Bath*

6.3.4 *Conical Flasks, Beakers*

6.3.5 *Petri Plates, Bacteriological Streaking Loop*

6.3.6 *Bacteriological Incubator*

6.3.7 *Microscope*

6.3.8 *Microscopic Slides*

6.3.9 *Cover Slips*

6.3.10 *Ice Bath*

6.3.11 *PCR Tubes,* of 0.2 ml

6.3.12 *Microfiltertips,* of capacities 10, 100 and 1 000 µl

6.3.13 *Variable Micropipettes,* of capacities 0.2, 10, 100 and 1000 µl

6.3.14 *PCR Machine* ,for running PCR master mix reaction

6.3.15 *Horizontal Electrophoresis System*

6.3.16 *Gel Documentation System*

6.3.17 *Hot Air Oven*

6.3.18 *Autoclave*

6.4 Procedure

6.4.1 *Testing for Clostridium botulinum*

6.4.1.1 Isolation of test organism and microscopic examination shall be done as per **8.2.1** and **8.2.2** of IS 5887 (Part 4).

6.4.1.2 *Confirmation test by polymerase chain reaction*

6.4.1.2.1 *Preparation of DNA template*

Mix 1 g of sample with 9 ml of TPGY medium and incubate under anaerobic conditions at 37 °C for 24 h. Take 1 ml of the culture and subject it for DNA extraction. DNA extraction shall be carried out using bacterial DNA extraction kit as per manufacturer's protocol.

6.4.1.2.2 *Primers to be employed*

The primers for confirming *Clostridium botulinum* are given in Table 3 and Table 4.

Table 3 Primers for Confirming *Clostridium botulinum* Type A, B, E and F

(*Clause* 6.4.1.2.2)

Toxin Type	Primer Sequence 5' – 3'	Product Size (bp)
A	For-5'-GGG CCT AGA GGT AGC GTA RTG-3' Rev-5'-TCT TYA TTT CCA GAA GCA TAT TTT-3'	101
B	For-5'-CAG GAG AAG TGG AGC GAA AA-3' Rev-5'-CTT GCG CCT TTG TTT TCT TG-3'	205
E	For-5'-CCA AGA TTT TCA TCC GCC TA-3' Rev-5'-GCT ATT GAT CCA AAA CGG TGA-3'	389
F	For-5'-CGG CTT CAT TAG AGA ACG GA-3' Rev-5'-TAA CTC CCC TAG CCC CGT AT-3'	543

Table 4 Primers for Confirming *Clostridium botulinum* Type C and D

(*Clause* 6.4.1.2.2)

Toxin Type	Primer Sequence 5' – 3'	Product Size (bp)	References
C and D	For-5'- TTT ATA CGA GAA TGT TCY G -3' Rev-5'- CAT TAT ATC CTG ATG TAT CC -3'	327	Prevot et al, 2007, Zoonoses and Public Health, 54:320-327.
C	For-5'- TCC TCG AGT TAC AAG CC -3' Rev-5'- CAG GAA AGG GTA TAT CTG -3'	169	
D	For-5'- TTA GAC TAT ACA GCA TCC C -3' Rev-5'- TAA CTT GTG GAC GAA TCC -3'	264	

6.4.1.2.3 *PCR master (mix)*

For detection of C. *botulinum* Type A, B, E and F

Carry out PCR in 25 µl reaction volume. PCR mix is as follows:

10X PCR buffer	:	2.5 µl
25 mM $MgCl_2$	:	2.5 µl
10 mM dNTPs	:	4.0 µl
10 µM Primer A For	:	0.75 µl
10 µM Primer A Rev	:	0.75 µl
10 µM Primer B For	:	0.75 µl
10 µM Primer B Rev	:	0.75 µl
10 µM Primer E For	:	0.75 µl
10 µM Primer E Rev	:	0.75 µl
10 µM Primer F For	:	0.75 µl
10 µM Primer F Rev	:	0.75 µl
5U/µl Taq polymerase	:	0.25 µl
DNA template	:	2 µl
PCR grade water	:	to make the 25 µl reaction volume

For detection of *C. botulinum* Type C and D

Carry out PCR in 25 µl reaction volume. PCR mix is as follows:

10X PCR buffer	:	2.5 µl
25 mM $MgCl_2$	:	2.5 µl
10 mM dNTPs	:	4.0 µl
10 µM Primer C and D For	:	1.25 µl
10 µM Primer C and D Rev	:	1.25 µl
10 µM Primer C For	:	1.25 µl
10 µM Primer C Rev	:	1.25 µl
10 µM Primer DFor	:	1.25 µl
10 µM Primer D Rev	:	1.25 µl
5U/µl Taq polymerase	:	0.25 µl
DNA template	:	2 µl
PCR grade water	:	to make the 25 µl reaction volume

6.4.1.2.3 *PCR cycling conditions*

For Detection of *C. botulinum* Type A, B, E and F

Use the following PCR cycles: 1 × 95°C for 15 min; 30 × (95°C for 0.5 min, followed by 56°C for 0.5 min, followed by 72°C for 1.5 min); 1 × 72°C for 7 min.

Initial Denaturation	Denature	Anneal	Extend	Final Extension	Hold
	30 cycles				
95°C for 15 min	95°C for 30 s	56°C for 30 s	72°C for 90 s	72°C for 7 min	4°C

For Detection of *C. botulinum* type C and D

Use the following PCR cycles: 1 × 95°C for 15 min; 40 × (94°C for 0.5 min, followed by 55°C for 0.5 min, followed by 72°C for 0.5 min); 1 × 72°C for 10 min.

Initial Denaturation	Denature	Anneal	Extend	Final Extension	Hold
	40 cycles				
95°C for 15 min	94°C for 30 s	55°C for 30 s	72°C for 30 s	72°C for 10 min	4°C

After PCR, 10 percent of the reaction volume of tracking dye (0.02 percent xylenecyanol, 0.02 percent bromophenol blue and 50 percent glycerol) is added to each PCR tube, and 10 µl loaded into wells on a 2 percent agarose gel in TAE and the gel electrophoresed for 45 min at 120 V followed by staining in ethidium bromide solution for visualization under UV light. A 100 bp ladder for size markers shall be included.

6.4.1.2.4 *Expression of results and calculations*

The PCR products showing bands of amplicon size corresponding to the primers used indicate sample positive for the stains of *Clostridium botulinum* as shown in Table 3.

6.4.2 *Testing for Clostridium chauvoei and Clostridium septicum*

6.4.2.1 *Isolation of test organism and microscopic examination*

Mix about 100 g of the material with about 1 000 ml of the sterile sodium chloride solution (0.9 percent). Decant the supernatant fluid and centrifuge. Suspend the packed sediment in about 3 ml of the sterile sodium chloride solution. Heat at 70 °C for 20 min in a water bath. Streak the heated sample on several freshly prepared blood agar plates and incubate anaerobically at 37 °C for 3 to 5 days. *Clostridium chauvoei,* if present, will develop in the form of irregularly round colonies 3 to 6 mm in diameter, effuse with entire or rhizoid edge. There will be no definite haemolytic zone, but the plate will be cleared slightly around the colonies. *Clostridium septicum* colonies will be larger, about 10 mm in diameter, irregularly round, effuse and having a cigarette-in-water appearance. Haemolysis will be of the beta type with prolonged incubation.

6.4.2.2 *Confirmation test by polymerase chain reaction*

6.4.2.2.1 *Preparation of DNA template*

Pick two to three suspected colonies and suspend in 200 µl of sterile normal saline. Subject them for DNA extraction using a bacteria DNA extraction kit as per the manufacturer's protocol.

6.4.2.2.2 *Primers to be employed*

The primers for confirming *Clostridium chauvoei* and *Clostridium septicum* are given in Table 5.

6.4.2.2.3 *PCR master mix*

Carry out PCR in 25 µl reaction volume. PCR mix is as follows:

10X PCR buffer	:	2.5 µl
25 mM $MgCl_2$	:	2.5 µl
10 mM dNTPs	:	4.0 µl
10 µM Primer For	:	0.5 µl
10 µM Primer Rev	:	0.5 µl
5U/µl Taq polymerase	:	0.25 µl
DNA template	:	2 µl
PCR grade water	:	to make the 25 µl reaction volume

6.4.2.2.4 *PCR cycling conditions*

Use the following PCR cycles: 1 × 95°C for 5 min; 30 × (95°C for 0.5 min, followed by 56°C for 0.5 min, followed by 72°C for 1.5 min); 1 × 72°C for 7 min.

Initial Denaturation	Denature	Anneal	Extend	Final Extension	Hold
	30 cycles ⟷				
95°C for 5 min	95°C for 30 s	56°C for 30 s	72°C for 90 s	72°C for 7 min	4°C

After PCR, 10 percent of the reaction volume of tracking dye (0.02 percent xylenecyanol, 0.02 percent bromophenol blue and 50 percent glycerol) is added to each PCR tube, and 10 µl loaded into wells on a 2 percent agarose gel in TAE and the gel electrophoresed for 45 min at 120 V followed by staining in ethidium bromide solution for visualization under UV light. A 100 bp ladder for size markers shall be included.

6.4.2.2.5 *Expression of results and calculations*

The PCR products showing bands of amplicon size of 522 bp indicate that the sample is positive for *Clostridium chauvoei* and 594 bp indicate that the sample is positive for *Clostridium septicum*.

6.4.3 *Testing for Clostridium perfringens*

6.4.3.1 *Isolation of test organism and microscopic examination*

When isolated according to **8.1.1** and **8.1.2** of IS 5887 (Part 4), the morphology described in **5.1** of IS 5887 (Part 4) is indicative of *Clostridium perfringens.*

6.4.3.2 *Confirmation test by polymerase chain reaction*

6.4.3.2.1 *Preparation of DNA template*

Pick two to three colonies from blood agar plate (*see* **5.2.11**) and suspend in 200 µl of sterile normal saline. DNA extraction shall be carried out using bacteria DNA extraction kit as per manufacturer's protocol.

6.4.3.2.2 *Primers to be employed*

The primers for confirming and toxin typing of *Clostridium perfringens* are given in Table 6.

6.4.3.2.3 *PCR master mix*

Carry out PCR using 25 µl reaction volume. PCR mix is as follows:

10X PCR buffer	:	2.5 µl
25 mM $MgCl_2$	:	2.5 µl
10 mM dNTPs	:	4.0 µl
10 µM Primer *cpa* For	:	0.5 µl
10 µM Primer *cpa* Rev	:	0.5 µl
10 µM Primer *cpb* For	:	0.5 µl
10 µM Primer *cpb* Rev	:	0.5 µl
10 µM Primer *cpb2* For	:	1.0 µl
10 µM Primer *cpb2* Rev	:	1.0 µl
10 µM Primer *etx* For	:	0.5 µl
10 µM Primer *etx* Rev	:	0.5 µl

Table 5 Primers for Confirming *Clostridium chauvoei* and *Clostridium septicum*

(*Clause* 6.4.2.2.2)

Target Gene	Primer Sequence 5' – 3'	Product Size (bp)
16-23s rRNA interspace region	For-5'-GAA AAT TGC ACA TGA ATT AAA-3'	522 bp: *C. chauvoei*
	Rev-5'-GGA TCA GAA CTC TAA ACC TTT CT-3'	594 bp: *C. septicum*

10 µM Primer *iap* For	:	0.5 µl
10 µM Primer *iap* Rev	:	0.5 µl
10 µM Primer *cpe* For	:	0.5 µl
10 µM Primer *cpe* Rev	:	0.5 µl
5U/µl Taq polymerase	:	0.25 µl
DNA template	:	2 µl
PCR grade water	:	to make the 25 µl reaction volume

6.4.3.2.4 *PCR cycling conditions*

Use the following PCR cycles: 1 × 95°C for 15 min; 40 × (94°C for 0.5 min, followed by 53°C for 1.5 min, followed by 72°C for 1.5 min); 1 × 72°C for 10 min.

Initial Denaturation	Denature	Anneal	Extend	Final Extension	Hold
	40 cycles ◄——►				
95°C for 15 min	94°C for 30 s	53°C for 90 s	72°C for 90 s	72°C for 10 min	4°C

After PCR, 10 percent of the reaction volume of tracking dye (0.02 percent xylenecyanol, 0.02 percent bromophenol blue and 50 percent glycerol) is added to each PCR tube, and 10 µl loaded into wells on a 2 percent agarose gel in TAE and the gel electrophoresed for 45 min at 120 V followed by staining in ethidium bromide solution for visualization under UV light. A 100 bp ladder for size markers should be included.

6.4.3.2.5 *Expression of results*

The PCR products showing bands of amplicon size corresponding to the primers used indicate sample positive for *Clostridium perfringens* as shown in Table 6.

7 TESTING FOR COLIFORM ORGANISMS

7.1 The coliform group of bacteria in this standard includes all aerobic, facultative aerobic, gram negative, non-spore forming rode capable of fermenting lactose with the production of acid and gas within 48 h at 37°C on solid or liquid media. They include a number of species of *Escherichia*, *Aerobacter*, *Klebsielia* and the *Paracolin bacilli*. The presence of these organisms gives a rough index of faecal contamination.

7.2 Medium

7.2.1 *Violet Red Bile Agar (VRBA)*

Prepare the medium preferably from dehydrated base from for from ingredients consisting of 0.3 percent yeast extract, 0.7 percent peptone, 0.15 percent bile salts, one percent lactose, 0.5 percent sodium chloride, 1.5 percent agar, 0.000 3 percent neutral red and 0.000 2 percent crystal violet meant for use in bacteriological work, in water, with final of *p*H 7.4 + 0.1. After complete dehydration, cool to 42 to 44°C before pouring in plates. After complete solidification of the medium in plate, add cover layer of the medium. Preferably prepare the medium shortly before use, otherwise sterilize by autoclaving at 121°C for 15 min before use.

Table 6 Primers for Confirming and Toxin Typing of *Clostridium perfringens*

(*Clause* 6.4.3.2.2)

Toxin Gene	Primer Sequence 5' – 3'	Product Size (bp)
cpa	For-5'-GCTAATGTTACTGCCGTTGA-3' Rev-5'-CCTCTGATACATCGTGTAAG-3'	324
cpb	For-5'-GCGAATATGCTGAATCATCTA-3' Rev-5'- GCAGGAACATTAGTATATCTTC-3'	195
cpb2	For-5'-AAATATGATCCTAACCAAMAA-3' Rev-5'- CCAAATACTYTAATYGATGC-3'	548
etx	For-5'-TGGGAACTTCGATACAAGCA-3' Rev-5'- AACTGCACTATAATTTCCTTTTCC-3'	376
iap	For-5'- AATGGTCCTTTAAATAATCC-3' Rev-5'- TTAGCAAATGCACTCATATT-3'	272
cpe	For-5'- TTCAGTTGGATTTACTTCTG-3' Rev-5'- TGTCCAGTAGCTGTAATTGT-3'	485

7.3 Quantitative Coliform Test

7.3.1 Melt sufficient violet red bile agar in boiling water, cool and hold in a water-bath at 45°C until needed. Slightly loosen screw caps on bottles before heating medium. Prepare 10^{o}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, dilutions of the feed samples in sterile phosphate buffered distilled water (diluent). For solid specimens, mixing and dilution is facilitated by homogenizing the specimen in the diluents with aid of a blender. Standard bacteriological transfer pipettes are used in making dilutions. Transfer 1 ml of well mixed dilution in corresponding previously labelled sterile petri dishes. Transferring 0.1 ml of dilution to a dish will increase the dilution factor by 10. For each dilution 2 plates are poured. Lift cover of dish only sufficiently to admit pipette.

7.3.2 Remove cap from bottle of violet red bile agar medium (which should not be above 45°C) and after flaming neck pour 10 to 12 ml of the melted medium into each dish. Immediately replace lid and mix medium and inoculums by gently rotating the dish first in one direction and then in the opposite. Care shall be taken not to splash medium over edge of dish. After the medium in the inoculated dishes has hardened, add aseptically about 3 to 4 ml more medium to completely cover the surface of the medium in the dish, tilt dish in various directions until the added medium entirely covers the surface and then allow harden. Count the colonies developing upon the medium after 24 h of incubation at 35 to 37°C, choosing only those plates having 30 to 300 colonies, which are dark red in colour, and have a diameter of at least 0.5 mm. Record count as coliform per ml or gram of product using the appropriate dilution factor.

8 TESTING FOR *SALMONELLA*

For testing *Salmonella*, the methods given in IS 5887 (Part 3) shall be employed.

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